Ponticoccus lacteus sp. nov. of the family *Rhodobacteraceae*, isolated from surface seawater

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A Gram-stain-negative, rod-shaped, non-motile, aerobic bacterium, strain JL351^T, was isolated from the surface seawater of the South China Sea. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain had a close relationship with members of the genera *Ponticoccus, Antarctobacter* and *Sagittula*, and the closest relative was *Ponticoccus litoralis* CL-GR66^T (with 96.56 % 16S rRNA gene sequence similarity). The polar lipids of strain JL351^T comprised diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, three unidentified aminolipids, three unidentified phospholipids and an unidentified glycolipid. The predominant isoprenoid quinone was Q-10. The major fatty acids were $C_{18:1}\omega7c$ (60.9 %), $C_{18:0}$ (13.7 %), $C_{16:0}$ (9.4 %), 11-methyl $C_{18:1}\omega7c$ (4.5 %), and $C_{12:1}$ 3-OH (4.4 %). The DNA G+C content was 66.2 mol%. Based on phenotypic, phylogenetic and genotypic data, strain JL351^T is considered to represent a novel species in the genus *Ponticoccus*, for which the name *Ponticoccus lacteus* sp. nov. is proposed. The type strain is JL351^T (=CGMCC 1.12986^T=JCM 30379^T).

Bacteria of the *Roseobacter* clade form a major lineage of the family *Rhodobacteraceae* within the class *Alphaproteobacteria* (Woese *et al.*, 1984). Organisms of this clade are widespread in marine environments and have been detected in a large variety of habitats such as coastal regions, sediments, sea ice and marine invertebrates (Buchan *et al.*, 2005; Wagner-Döbler & Biebl, 2006; Tang *et al.*, 2010). The cultivated bacteria within this lineage are closely related to environmental clones, so studies on this group are important for understanding marine bacterial ecology and physiology (Wagner-Döbler & Biebl, 2006).

Here, strain JL351^T was obtained from a surface seawater sample during a South China Sea cruise located at longitude 111° 00′ E and latitude 20° 59′ N. A total of 100 µl seawater supplemented with 15 % (v/v) glycerol was taken and spread on a marine agar 2216 (MA; Becton Dickinson) plate, which was then incubated at 28 °C for one week and subsequently purified as single colonies.

Cell morphology was observed by transmission electron microscopy (JEM-2100; JEOL). Cell motility was tested via the semi-solid puncture method (Dong & Cai, 2001). The Gram reaction was performed according to the method described by Gerhardt *et al.* (1994). Poly-β-hydroxybutyrate (PHB) granules were observed by epifluorescence microscopy (BX51; Olympus) after Nile blue A staining (Ostle & Holt, 1982).

The range of temperature for growth was observed by assessing changes in OD_{600} over the incubation period (up to 3 days) in marine broth 2216 (MB; Becton Dickinson) at temperatures of 4, 10, 15, 20, 25, 30, 35 and 40 °C (at pH 7.8, 2.0 % NaCl). The pH range for growth was tested by adjusting the final pH to 5.0, 6.0, 7.0, 8.0, 9.0 10.0 and 11.0 (at 2.0 % NaCl, 28 °C) with appropriate buffers (Na_2HPO_4/NaH_2PO_4 for pH 5.0–7.0 and $Na_2CO_3/NaHCO_3$ for pH 8.0–11.0). The optimum NaCl concentration for growth was tested at 0, 0.5 and 1.0–11.0 % NaCl (intervals of 1 %) (at pH 7.8, 28 °C) in a rich organic (RO) medium (Yurkov *et al.*, 1999) that contained 1 g Bacto peptone, 1 g yeast extract and 1 g sodium acetate per litre artificial seawater (0.5 g $MgSO_4.7H_2O$, 0.3 g KCl, 0.05 g $CaCl_2.2H_2O$, 0.3 g NH_4Cl, 0.3 g K_2HPO_4) (Yurkov *et al.*, 1999).

Oxidase and catalase activity, and hydrolysis of DNA (0.2 %, w/v) and starch (0.2 %, w/v) were determined according to the methods of Smibert & Krieg (1994). Sole carbon-source utilization was tested by using Biolog GN2 microplates (Rüger & Krambeck, 1994). Other phenotypic and enzymic characterizations of strain JL351^T were conducted using API 20E, API 20NE and API ZYM kits (bioMérieux) according to the manufacturers' instructions. Susceptibility to antibiotics was tested by using the disc-diffusion plate method (Fraser & Jorgensen, 1997) with

Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JL351^T is DQ104407.

Two supplementary figures are available with the online Supplementary Material.

discs containing ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) vancomycin (30 μg), gentamicin (10 μg) and polymyxin B (30 μg). Discs impregnated with antibiotics were placed on the MA plate surfaces, and the bacterial cultures (200 μl) spread on the plate were observed after 3 days at 28 °C. All the above tests were performed on strain JL351^T and the following reference strains: *Ponticoccus litoralis* CL-GR66^T (=DSM 18986^T), *Sagittula stellata* E-37^T (=DSM 11524^T) and *Antarctobacter heliothermus* EL-219^T (=DSM 11445^T).

Strain JL351^T and reference strains were incubated on MA medium in order to extract the fatty acids. According to the quadrant streak method (Sasser, 1990), the physiological age of the culture was standardized by using the third sector from the quadrant streak on the plate and then fatty acids were analysed as described by Fraser & Jorgensen (1997). Polar lipids were extracted using a chloroform/methanol system and analysed using two-dimensional TLC with DSM 18986^T as a reference strain, as described by Kates *et al.* (1972). Merck silica gel 60F_{254} aluminium-backed thin-layer plates were used in TLC analysis. The DNA G+C content was determined by the HPLC method of Mesbah *et al.* (1989). Isoprenoid quinones were extracted by using the two-stage method of Tindall (1990a, b) and analysed by HPLC.

Genomic DNA was extracted using a TIANamp Bacteria DNA kit (DP302; Tiangen Biotech). Cells were incubated in MB medium for 2 days at 28 °C. The DNA purity and concentration were assessed with a NANODROP 2000 spectrophotometer (Thermo Scientific). The 16S rRNA gene was amplified by PCR using universal bacterial primers 27F and 1492R (Embley, 1991). Afterwards, the sequence was submitted to the GenBank database (NCBI) and compared with sequences available in GenBank by using the EzTaxon-e server (Kim *et al.*, 2012) to determine their approximate phylogenetic affiliations. Phylogenetic trees were generated by MEGA version 5 software using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms (Tamura *et al.*, 2011) after sequence alignment was performed with BioEdit 7.0.9 (Hall, 1999).

Cells of strain JL351^T were aerobic, cream, rod-shaped, non-motile and Gram-stain-negative. The cells were approximately 0.5–0.8 μm wide and 0.7–1.5 μm long (Fig. S1, available in the online Supplementary Material). PHB granules were observed. Bacteriochlorophyll *a* was not detected. Other phenotypic characteristics of strain JL351^T

Table 1. Differential characteristics of strain JL351^T and strains of three related taxa within the family *Rhodobacteraceae*

Strains: 1, JL351^T; 2, *P. litoralis* DSM 18986^T; 3, *S. stellata* DSM 11524^T; 4, *A. heliothermus* DSM 11445^T. +, Positive; −, negative; W, weakly positive; C, coccus; R, rod.

Characteristic	1	2	3	4
Cell shape	R	C–R[a]*	R[b]	R[c]
Cell size (wide × long) (μm)	0.5–0.8 × 0.7–1.5	0.5–0.8 × 0.8–1.2[a]	0.9 × 2.3[b]	1.2 × 2.5[c]
Motility	−	−	−	+
Major fatty acids (%)				
$C_{16:0}$	9.4	5.4	9.9	4.9
$C_{18:1}\omega7c$	60.9	80.0	70.0	78.1
$C_{18:0}$	13.7	3.7	2.4	2.1
11-Methyl $C_{18:1}\omega7c$	4.4	5.1	2.9	4.9
$C_{12:1}$ 3-OH	4.4	0.8	3.8	4.1
$C_{19:0}$ cyclo $\omega8c$	1.0	−	4.1	3.7
Enzymic activity (API ZYM)				
Alkaline phosphatase, acid phosphatase	+	+	+	−
Naphthol-AS-BI-phosphohydrolase	+	+	−	−
α-Galactosidase, β-galactosidase	+	−	−	−
Acid production from (API 20E):				
D-Glucose	−	+	−	−
Sucrose	+	+	W	−
Melibiose				
Assimilation of (API 20NE):				
L-Arabinose	+	+	+	−
Hydrolysis of:				
Urea	−	−	+	+
L-Tryptophan	+	−	−	−

*Data taken from: *a*, Hwang & Cho (2008); *b*, Labrenz *et al.* (1998); *c*, González *et al.* (1997).

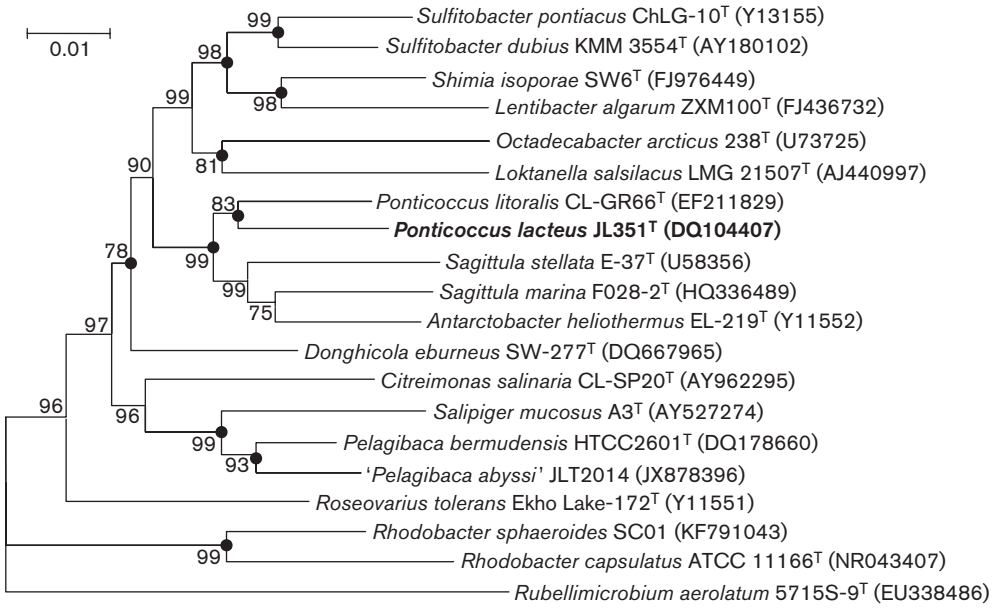

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain JL351^T and members of representative species of the genus *Ponticoccus* and related genera. Bootstrap percentages are based on 1000 replications and only values >50 % are shown. *Rubellimicrobium aerolatum* 5715S-9^T was used to root the tree. Filled circles indicate that the corresponding nodes were recovered in the trees generated with the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms. Bar, 0.01 substitutions per nucleotide position.

are given in the species description and in Table 1. Growth was supported in the temperature range between 15 and 40 °C (optimum, 30 °C), at pH 7.0–10.0 (optimum, pH 8.0) and with 0.5–9.0 % (w/v) NaCl (with good growth across the range of 1.0–4.0 %).

Oxidase and catalase activities of strain JL351^T were both positive. The predominant fatty acid was $C_{18:1}\omega7c$ (60.9 %), followed by $C_{18:0}$ (13.7 %), $C_{16:0}$ (9.4 %), 11-methyl $C_{18:1}\omega7c$ (4.5 %) and $C_{12:1}$ 3-OH (4.4 %).The major isoprenoid quinone was Q-10. The DNA G+C content was 66.2 mol%. The polar lipids comprised diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, three unidentified aminolipids, three unidentified phospholipids and an unidentified glycolipid (Fig. S2).

Strain JL351^T revealed a clear affiliation with the *Roseobacter* clade in the family *Rhodobacteraceae* (Fig. 1). Based on the phylogenetic tree, strain JL351^T was closely related to *P. litoralis* CL-GR66^T (96.56 % 16S rRNA gene sequence similarity), *S. stellata* E-37^T (96.50 %) and *A. heliothermus* EL-219^T (96.47 %). Several phenotypic and chemotaxonomic characteristics could be used to distinguish between JL351^T and other similar strains (Table 1). Strain JL351^T could be differentiated from *S. stellata* DSM 11524^T from its absence of blebs or vesicles and polysaccharide fibrils, as well as its lack of cell polarity and its naphthol-AS-BI-phosphohydrolase activity (González *et al.*, 1997). These taxa also differed in their optimal temperature for growth (30 °C for JL351^T and 16–26 °C for *A. heliothermus* DSM 11445^T). In addition, JL351^T lacked flagella, but cells of *A. heliothermus* DSM 11445^T had flagella and were motile (Labrenz *et al.*, 1998). Furthermore, the inability of JL351^T to hydrolyse urea also differentiated this strain from *A. heliothermus* DSM 11445^T and *S. stellata* DSM 11524^T. JL351^T and *P. litoralis* DSM 18986^T shared some common characteristics with strain JL351^T, including acid production from sucrose and melibiose as well as being positive for alkaline phosphatase and acid phosphatase, but strain JL351^T could be distinguished clearly from *P. litoralis* DSM 18986^T by characteristics such as the presence of diphosphatidylglycerol, $C_{19:0}$ cyclo $\omega8c$, and α-galactosidase and β-galactosidase activities, and its resistance to gentamicin (Fig. S2, Table 1). On the basis of phylogenetic analysis and phenotypic characteristics, strain JL351^T is considered to represent a novel species of the genus *Ponticoccus*, for which the name *Ponticoccus lacteus* sp. nov. is proposed.

Description of *Ponticoccus lacteus* sp. nov.

Ponticoccus lacteus (lac.te′us. L. masc. adj. *lacteus* milky).

Cells are Gram-stain-negative, rod-shaped, aerobic, non-motile, oxidase- and catalase- positive. Cells form creamy, uniformly circular, convex colonies on MA medium after incubating for 2 days at 28 °C. Both starch and DNA can be hydrolysed. The dominant cellular fatty acid is $C_{18:1}\omega7c$. The isoprenoid quinone is Q-10. In assays with the API ZYM system, acid phosphatase, alkaline phosphatase, esterase

(C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase activities are present, but trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and β-fucosidase activities are absent. With the API 20E test, acid is formed from D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose and melibiose. H_2S production and hydrolysis of urea, L-lysine and L-ornithine are not detected. The following substrates are oxidized as carbon sources from the results of Biolog GN2: methyl β-D-glucoside, α-lactose, L-arabinose, maltose and β-hydroxybutyric acid. Cells are sensitive to ampicillin, chloramphenicol, erythromycin, kanamycin, penicillin G, streptomycin, tetracycline and vancomycin, but are resistant to gentamicin.

The type strain, JL351^T (=CGMCC 1.12986^T=JCM 30379^T), was isolated from surface seawater in the South China Sea. The DNA G+C content of the type strain is 66.2 mol%.

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